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In Situ-Generated Thrombin Is the Only Enzyme That Effectively Activates Factor VIII and Factor V in Thromboplastin-Activated Plasma

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We investigated the activation of the nonenzymatic protein cofactors factor VIII and factor V in plasma when coagulation was initiated by thromboplastin. With sensitive bioassays, we were able to measure specifically the generation of activated factor VIII and activated factor V in plasma. Our results showed that when plasma was triggered with a relatively high concentration of thromboplastin, factor VIII and factor V were completely activated at the clotting time of plasma. However, when the generation of thrombin, but not that of factor Xa, was delayed by addition of hirudin to the plasma, factor Va was generated only at the time thrombin generation overcame the hirudin inhibition. In addition, generation of factor VIIIa correlated with thrombin generation and not with factor Xa generation. Furthermore, addition of large amounts of factor Xa to hirudinized plasma did not show detectable factor VIII or factor V activation. We concluded that in plasma activated with thromboplastin the enzyme responsible for activation of factor V and factor VIII is thrombin, not factor Xa.

CLOTTING OF PLASMA is the result of a series of enzymatic reactions acting in a cascade system.[1] Starting either from the intrinsic or extrinsic coagulation pathway, the coagulation system comprises a number of feedback reactions (eg, generation of thrombin through the intrinsic pathway proceeds only at a considerable rate after the nonenzymatic protein cofactors factor V and factor VIII have been activated.[2,3] Activated factor VIII serves as an essential cofactor for activation of factor X by factor IXa, whereas factor Va plays the same role in the conversion of prothrombin into thrombin by factor Xa.

Conversion of factor VIII into its activated form is believed to proceed principally through the proteolytic activity of thrombin,[4] although factor Xa is also considered a possible important activator of factor VIII.[4-6] Activation of factor VIII by factor IXa has been demonstrated in a purified system. However, the factor IXa concentrations used in this study exceeded the plasma factor IX concentration, making it unlikely that factor IXa is physiologically important for this activation.[7]

Thrombin and factor Xa have been demonstrated to activate factor V.[8,9] However, the thrombin-catalyzed activation of factor V appeared to be, on a molar basis, 100-fold faster than the factor Xa-catalyzed reaction.[9] Proteases present in platelets and endothelial cells have also been shown to activate factor V, although it is not clear if these activation reactions occur under physiologic conditions.[10-12]

The relative importance of these activators for in vivo hemostasis is not clear. Thus, although a number of enzymes have been described to activate factor VIII and factor V in purified systems, the relative catalytic efficiencies of these enzymes in plasma are unknown. Therefore, we investigated generation of activated factor V and activated factor VIII under more physiologic conditions, ie, thromboplastin-activated plasma. With very specific bioassays, we were able to follow the generation of factor Va and factor VIIIa during plasma coagulation. With hirudin, we were able to separate thrombin generation from factor Xa generation and clearly demonstrated that in this system thrombin is the only enzyme responsible for activation of both factor V and factor VIII.

MATERIALS AND METHODS

The synthetic peptide substrates Bz-Ile-Glu-(piperidyl)-Gly-Arg-*p*-nitro-anilide hydrochloride (S-2337) and *H*-D-phenylalanyl-L-pipecolyl-L-arginine-*p*-nitro-anilide dihydrochloride (S-2238) were purchased from AB Kabi, Stockholm. Ovalbumin was a product of Sigma Chemical, St Louis. All reagents used were of the highest grade commercially available. Human brain thromboplastin was prepared by the method of Owren and Aas.[13] It was homogenated in a Potter Elvehjem homogenizer for three minutes, centrifuged at 2,000 g for 15 minutes, and stored at −80°C. Its concentration was adjusted with Tris-saline (50 mmol/L Tris, 180 mmol/L NaCl, pH 7.9) to give a prothrombin time of 13 s.

Proteins. Recombinant desulfato-hirudin variant 1 (CGP 39393) was provided by Dr Wallis, Ciba-Geigy, Horsham, England. All bovine clotting factors were prepared and quantitated as previously described.[2,14,15] Human factor Xa was purified according to the method of Mertens and Bertina.[16] Its molar concentration was determined by active-site titration with *p*-nitrophenyl *p*-guanidinobenzoate hydrochloride.[15]

Phospholipid. Phospholipid vesicles were made from a mixture of phosphatidylserine (PS) and phosphatidylcholine (PC) as previously described.[15] Vesicles composed of 25 molar percent PS and 75 molar percent PC were used throughout the experiments.

Preparation of human normal plasma. Blood from healthy donors was collected on 0.13 mol/L trisodium citrate: nine parts blood to one part citrate solution. The blood was then centrifugated twice at room temperature for 15 minutes at 3,000 g. A third centrifugation was performed at 4°C for one hour at 23,000 g. The obtained platelet-free plasma was stored at −80°C.

Thromboplastin-induced factor Xa, factor Va, factor VIIIa, and thrombin generation in platelet-free plasma. To 0.13 mL citrated human plasma, 9 μL Tris buffer (50 mmol/L Tris, 180 mmol/L NaCl, 0.5 mg ovalbumin/mL, pH 7.9) with or without hirudin was added and incubated for four minutes in a flat-bottom plastic tube while the mixture was stirred. Clotting was initiated by addition of 11 μL Tris buffer containing 0.27 mol/L $CaCl_2$ and human brain thromboplastin. Timed samples (10 μL) were removed from incubations and assayed for either factor Va, factor Xa, or thrombin activity.

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Factor Va assay. Factor Va was assayed in plasma samples, diluted 1:200 in Tris buffer (pH 7.9) containing 5 mmol/L $CaCl_2$ and 0.5 μg heparin/mL. Ten microliters of the diluted sample was added to a cuvette containing 13 pmol/L factor Xa, 5 mmol/L $CaCl_2$, and 0.05 mmol/L phospholipid (25 molar percent phosphatidylserine and 75 molar percent phosphatidylcholine) in Tris buffer. After four minutes, thrombin generation was initiated by addition of prothrombin (0.2 μmol/L final concentration in 125 μL). The reaction was stopped after two minutes by addition of 325 μL Tris buffer containing 20 mmol/L EDTA. The amount of thrombin was measured in a dual-wavelength spectrophotometer using S-2238 as described previously.[17]

Factor VIIIa assay. Factor VIIIa was assayed in plasma samples diluted 1:40 directly into the assay mixture containing 100 nmol/L bovine factor IXa, 0.02 mmol/L phospholipid, 5 mmol/L $CaCl_2$, and 0.5 mg/mL ovalbumin in Tris buffer (pH 7.9). The reaction was started after 15 seconds on addition of bovine factor X (final concentration 0.5 μmol/L). At 30 and 90 seconds, 50 μL was removed and assayed for factor Xa activity with the chromogenic substrate S-2337. From these two time points, the rate of factor X activation was determined to be linear with the added amounts of factor VIIIa.

Factor Xa assay. Factor Xa was assayed in plasma samples diluted 1:200 in Tris buffer (pH 7.9). Ten microliters of the diluted sample was added to a cuvette containing 0.6 nmol/L factor Va, 0.05 mmol/L phospholipid, and 5 mmol/L $CaCl_2$ in Tris buffer. After four minutes, prothrombin (1.5 μmol/L final concentration in 50 μL) was added, and the assay was continued as described for factor Va.

Thrombin assay. Undiluted plasma samples (5 μL) were added to a cuvette containing 495 μL Tris buffer, 20 mmol/L EDTA, and 0.25 mmol/L S-2238. The thrombin activity was calculated from the change in absorbance as described previously.[17]

RESULTS

Factor VIIIa bioassay. To determine activated factor VIII in plasma, a highly sensitive and specific bioassay was developed making use of the activity of factor VIIIa as an essential cofactor in activation of factor X by factor IXa in the presence of phospholipids and calcium. By judicious choice of the concentrations of the reaction components, we could measure factor VIIIa activity without interference of nonactivated factor VIII. Figure 1 shows the calibration curve constructed from a series of plasma dilutions activated with thrombin (50 nmol/L). Assuming that the plasma concentration of factor VIII is ~1 nmol/L,[12,18] this assay is shown to be linear up to 40 pmol/L factor VIIIa and concentrations as low as 5 pmol/L can be easily determined. The intersection at the ordinate represents the activity of the assay without factor VIII(a). Figure 1 shows the activity of plasma not activated with thrombin (ie, the baseline value of the assay). Control experiments with factor VIII-deficient plasma showed that factor Xa generation in our assay occurred only in the presence of activated factor VIII.

Effect of hirudin on thrombin and factor Xa generation. To investigate the relative importance of thrombin and factor Xa to activation of factor V and factor VIII, we first had to obtain in our plasma system nonoverlapping thrombin and factor Xa generation curves. Therefore, we incubated plasma with hirudin and activated it using a thromboplastin concentration of 1/30 (vol/vol). Thrombin generation and factor Xa generation were determined as

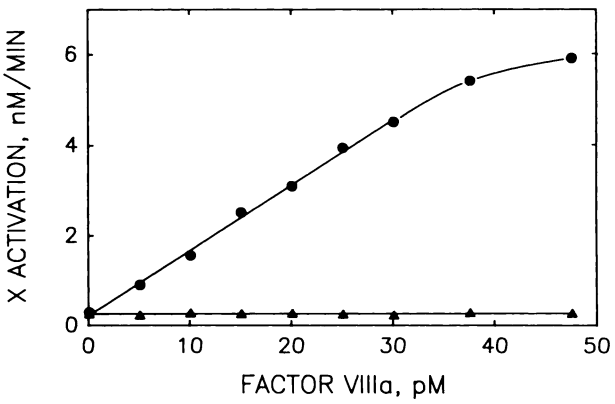

Fig 1. Calibration curve for factor VIIIa (●). Human normal plasma was diluted to obtain the concentrations of factor VIII as indicated, assuming that the concentration of plasma factor VIII is 1 nmol/L. The (diluted) plasma samples were activated with 50 nmol/L thrombin, and the activity was determined as described in the Materials and Methods section. The activity of plasma not activated with thrombin was also determined (▲).

described in the Materials and Methods section. Figure 2 shows the effect of the addition of different amounts of hirudin on thrombin and factor Xa generation. The thrombin generation curve without hirudin is characterized by a very short lag period followed by an explosive thrombin generation. The amidolytic activity decayed rapidly until a plateau was reached. This residual activity is the result of the formation of a thrombin–α_2-macroglobulin complex.[19] Figure 2A shows that the more hirudin present, the more the thrombin generation is delayed, and the maximum amount of amidolytic active thrombin formed is also decreased. Factor Xa generation and inactivation, as shown in Fig 2B, is not affected by hirudin.

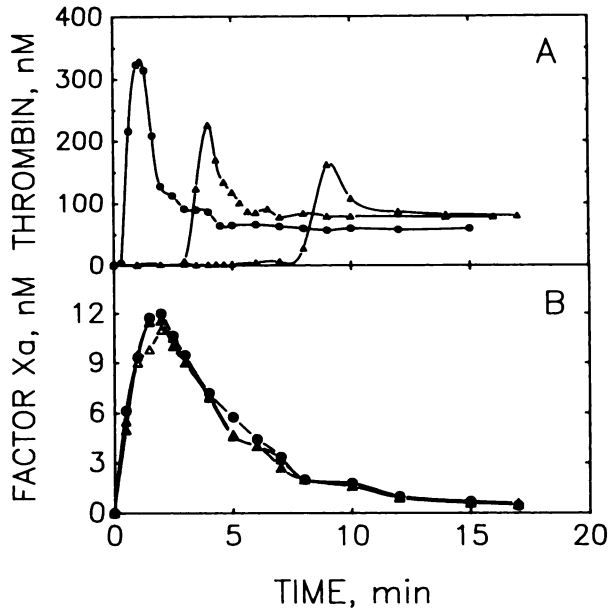

Fig 2. Effect of hirudin on thrombin generation (A) and factor Xa generation (B) in thromboplastin-activated plasma. Concentrations of hirudin used were 0 (●), 300 (▲), and 450 nmol/L (△).

Effect of hirudin on factor Va and factor VIIIa generation. Figure 3A shows factor Va generation curves as obtained when plasma was activated with thromboplastin with varying amounts of hirudin added. Apparently, factor Va generation is closely correlated with thrombin generation but not at all with factor Xa generation. As in the case of thrombin generation, increasing amounts of hirudin caused a prolongation of the lag-phase of factor Va generation. Despite a drastic decrease of the thrombin peak activity with increasing hirudin, maximal factor Va activities could be generated. Like the factor Va generation curves, the factor VIIIa generation curves (Fig 3B) showed a very rapid activation phase followed by a decay of factor VIIIa activity. In protein C-depleted plasma, factor Va was not inactivated.[20] Whether rapid decay of factor VIIIa is also due to inactivation by activated protein C remains to be established. Factor VIIIa generation is closely correlated with thrombin generation but not with factor Xa generation.

Exogenous factor Xa. To discover whether factor V and factor VIII could be activated by factor Xa in plasma at a concentration approaching the amount of factor X present in plasma, we added 100 nmol/L purified human factor Xa to plasma incubated for eight minutes with thromboplastin (1/30 vol/vol), $CaCl_2$ (20 mmol/L), and hirudin (2 μmol/L). Before addition of factor Xa, no factor Va, factor VIIIa, or thrombin activities could be detected. The endogenous factor Xa concentration was 2 nmol/L. After addition of factor Xa, samples were removed and assayed for factor Va, factor VIIIa, and thrombin. No amidolytic thrombin activity could be detected. Up to five minutes after addition of factor Xa, the amounts of factor Va and VIIIa were below the detection limits of the assays, 0.2 pmol/L and 2 pmol/L, respectively (ie, <1% of the plasma factor V and <5% of the plasma factor VIII was activated.

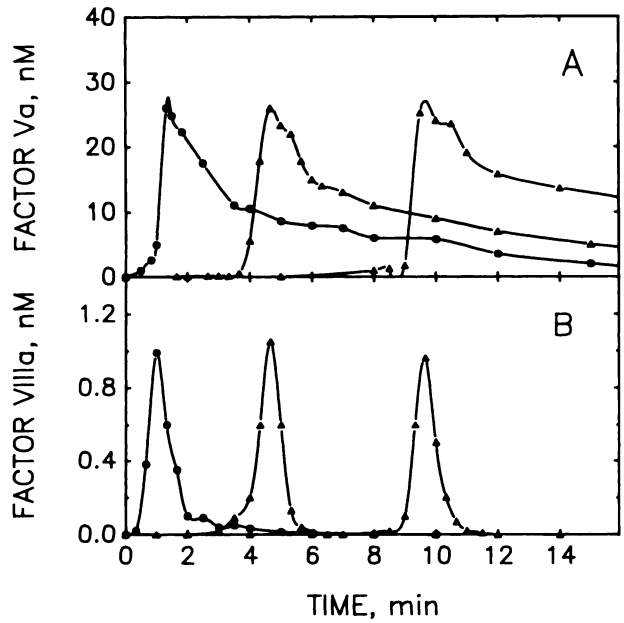

Fig 3. Effect of hirudin on factor Va generation (A) and factor VIIIa generation (B) in thromboplastin-activated plasma. Concentrations of hirudin used were 0 (●), 300 (▲), and 450 nmol/L (△).

DISCUSSION

Investigators have explained their findings regarding the effects of the anticoagulant drug heparin on the intrinsic pathway of blood coagulation by assuming that small amounts of factor Xa are able to activate factor VIII and factor V.[21-23] Although thrombin might be the most efficient activator of both cofactors,[12] thrombin is also the end product of the coagulation process. Therefore, a (partial) activation of factor V or factor VIII by factor Xa earlier in coagulation could amplify thrombin generation by inducing small amounts of thrombin which then through a positive feedback mechanism in turn activates factor VIII and factor V. In the case of heparin, when thrombin activity is blocked, factor Xa activation of factors V and VIII may become an important amplification reaction.[23] However, to our knowledge, that factor VIII and factor V activation by factor Xa is indeed essential to attain "physiologic" rates of factor Xa and thrombin generation in plasma has never been demonstrated.

In this study, we determined the relative contribution of factor Xa and thrombin to activation of the nonenzymatic protein cofactors factor VIII and factor V in plasma. Preliminary studies on the intrinsic pathway of blood coagulation revealed that factor Xa generation was strongly controlled by specific thrombin inhibitors like hirudin. Because thrombin and factor Xa generation cannot be separated, we could not assess the relative contribution of thrombin and factor Xa generation to activation of factor V and factor VIII when plasma clotting was initiated by addition of factor IXa (T. Lindhout and J. Pieters, unpublished observations).

Therefore, the plasma was triggered with thromboplastin at a concentration high enough to limit the activation reactions to the extrinsic pathway.[20] We developed a very specific assay to monitor generation of factor VIIIa in plasma, making use of the fact that (human) factor VIIIa is able to catalyze (bovine) factor X activation by (bovine) factor IXa in the presence of phospholipids and calcium. A sensitive assay for determination of factor Va generation in plasma was developed during earlier studies.[20]

Generation of factor Va in thromboplastin-activated plasma follows generation of thrombin and shows a maximum at ~ one minute. However, because factor Xa is also generated at that time, it is not clear whether thrombin or factor Xa is the activator of factor V in this system. To obtain a thrombin generation independent of free factor Xa generation, we added hirudin to the plasma in several concentrations. The thrombin generation curve obtained was indeed dependent on the hirudin concentration used, whereas the factor Xa generation curve was not influenced by hirudin. When we determined the generation of factor Va in this hirudinized plasma, it appeared to parallel the generation of thrombin but not that of factor Xa. Moreover, addition of a high amount of factor Xa to thromboplastin-activated plasma containing hirudin (2 μmol/L) did not cause any activation of factor V. Factor VIIIa generation was also monitored and entirely paralleled thrombin generation. Addition of a high amount of factor Xa did not induce factor VIIIa generation.

The results of our study using plasma as a physiologic

environment for coagulation and thromboplastin as a clotting trigger show that under these conditions thrombin is the only activator of the factors V and VIII. The mechanism of plasma clotting evolving from our results is one in which factor X is activated by the TF factor VII(a) complex. This initially present factor Xa is able, at a low rate, to convert prothrombin into thrombin, which then is able to activate factor VIII and factor V. Only then are sufficient amounts of thrombin generated to ensure proper clotting. The conclusions derived from this study once again emphasize the central role of thrombin in the overall process of in vitro blood coagulation.

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